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Applicants: Jason Francis Conaty et al.

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For : MINIRIBOZYMES ACTIVE AT LOW MAGNESIUM ION CONCENTRATES

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July 26, 2004

Commissioner for Patents
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Sir:

TRANSMITTAL OF CERTIFIED COPY OF EARLIER FILED FOREIGN APPLICATION AND CLAIM TO PRIORITY PURSUANT TO 35 U.S.C. §119

Applicants submit herewith, as **Exhibit A**, a certified copy of Australian Provisional Application No. PP 7951/98, which is cited in applicants' Declaration pursuant to 37 C.F.R. §1.63.

Applicants hereby claim the benefit of the December 24, 1998 filing date pursuant to 35 U.S.C. §119 and 37 C.F.R. §1.55(a).

Respectfully submitted,

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I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PP 7951 for a patent by COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION as filed on 24 December 1998.

CERTIFIED COPY OF PRIORITY DOCUMENT

WITNESS my hand this
Fourteenth day of July 2004

J. Billingsley

JULIE BILLINGSLEY
TEAM LEADER EXAMINATION
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Commonwealth Scientific and Industrial Research Organisation

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Miniribozymes active at low magnesium ion concentrations"

The invention is described in the following statement:

MINIRIBOZYMES ACTIVE AT LOW MAGNESIUM ION CONCENTRATIONS

FIELD OF THE INVENTION

This invention relates to minimised ribozymes, herein referred to as "miniribozymes", and in particular it relates to a class of miniribozymes which has been selected on the basis of the very high cleavage rates by the members of the class at low Mg^{2+} concentration. This invention also extends to compositions comprising these miniribozymes, to transfer vectors and host cells as well as methods of cleaving a target RNA in a subject and other methods of use of these miniribozymes.

Throughout this application, various references are cited in brackets. The full citations may be found in the numerical list of references immediately following the Example(s). These publications are hereby incorporated by reference into the present application.

BACKGROUND OF THE INVENTION

There is currently much interest in the catalytic potential of RNA both as a candidate molecule for precellular evolution and as gene targeted therapeutics. To date there are in excess of 30 distinct RNA motifs known to perform catalysis in the absence of protein. These include ribozymes derived from natural RNAs and also those produced by *in vitro* selection (1). The reactive capacity of any polynucleotide is a function of the chemistry of the individual nucleotides and the secondary and tertiary structure of the polymer, both of which are affected by coordination with metal ions (2,3). Structure within an oligonucleotide can be viewed as a direct function of the sequence identity. The catalytic versatility of a nucleic acid is therefore amenable to combinatorial studies, such as *in vitro* selection, involving the artificial manipulation of a discrete sequence space in response to selective pressure.

The 2' hydroxyl of ribonucleic acids is a crucial functional entity permitting nucleophilic attack by the deprotonated form on an adjacent bridging phosphate ester leading to hydrolysis (giving 5' OH and 2'-3' cyclic phosphate ends) via a pentacoordinate transition state (4-7). Enzymes of either nucleic acid or protein composition that catalyse reactions of phosphates are invariably metal ion dependent (8). Metals have been recruited by biology for this purpose presumably because phosphates are a favourable metal ion ligand, and because coordination with metals withdraws electron density from the phosphorus centre, making it more susceptible to nucleophilic attack. Metal ions may also assist the deprotonation of the attacking nucleophile and assist the stabilisation of the developing negative charge on the leaving group (8-10).

The hammerhead ribozyme was first identified as a self (*cis*) cleaving sequence found in a number of small, circular, RNA pathogens (virusoids and viroids) found in plants, and a satellite RNA found in newt (11). Its consensus structure consists of three helical regions which form at their junction, a conserved bed of 15 nucleotides. The bulk of the conserved nucleotides can be located on a single oligoribonucleotide constituting an enzymatic entity capable of cleaving multiple substrates (12,13). Ribozymes designed accordingly can be directed in *trans* against any RNA substrate containing an endogenous 5' UH (where H = C, U, or A) (14-16). There is significant interest in these enzymes because they offer a means of specifically inactivating deleterious RNA, eg. viral or oncogenic mRNAs, and thereby ameliorating disease.

Hammerhead cleavage of an RNA phosphodiester bond exhibits divalent metal ion dependence (17) typical of this form of catalysis in nature. Generally the metal ion is proposed to act both structurally to augment and direct specific helical interactions, and as a catalytic co-factor functional in the chemical step of the reaction (2,9,18). Mg^{2+} is known to perform both these roles effectively. Bassi et al (19-21) report that there is a two stage folding process leading to formation of the ground state, each with a specific Mg^{2+} requirement. Once the ground state is obtained, Mg^{2+} is thought to play

a role in activating the 2' OH nucleophile of C17, either by direct coordination, or by providing an appropriately positioned basic group (Mg-OH), to effect deprotonation, promoting nucleophilic attack on the adjacent phosphorus (22-24).

The role of helix II in the hammerhead ribozyme has been investigated in several deletion studies (25-28). The presence of Watson-Crick base pairing in this region is thought to stabilise the active conformation of the conserved nucleotides stacked above it (28). The role of helix II is therefore seemingly to limit the number of steric possibilities closer to the cleavage site. This effect is no doubt variable and will depend on the composition of nucleotides between positions A9 and G12. Crystal structures for the hammerhead ribozyme show proximity between helix I and helix II (29,30). These structures suggest a plausible interaction (perhaps Mg^{2+} mediated) between helix I and helix II. Whilst this interaction is remote from the cleavage site, it affects the global architecture of the molecule, and thus the cleavage rate. It has been speculated that the interaction between helix I and II may in fact stabilise an inactive conformation (31). The truncation of helix I appears to amend this interaction and allows higher rates of catalysis to be observed (31). Minimisation strategies involving helix II therefore might offer an alternative means of circumventing or closing down this particular equilibrium pathway, and thereby improve the catalytic outcome.

Miniribozymes are derivatives of the hammerhead ribozyme where helix II has been replaced by a linker with a single Watson-Crick base pair (32). This minimisation strategy has created a novel structural format. Whilst the full length ribozyme has been subject to selection over evolutionary time, size constrained, *trans* cleaving ribozymes have not been exposed to selection in nature. Minimised ribozymes have been shown to cleave long RNAs more efficiently than full length hammerheads (27), and could therefore provide improved trans cleaving activity in a cellular environment.

The work leading to the present invention has included *in vitro* optimisation of the novel miniribozyme structure. In particular, *in vitro* selection was used to search

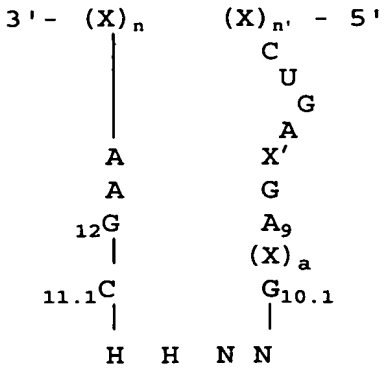
an 18 nt RNA sequence space corresponding in size to a miniribozyme (Figure 1). The aims were primarily to identify all motifs within this size constrained domain, capable of supporting Mg^{2+} dependent phosphodiester cleavage of a 29mer RNA substrate containing a 13 nt segment of human IL-2 mRNA. Subsequently, the aim was to direct the active component of this population towards optimum catalytic efficiency at low concentrations of Mg^{2+} (0.5 - 2 mM) such as occur intracellularly (33). This work has shown that the active population consisted almost entirely of molecules containing conserved nucleotides conforming to recognised hammerhead motifs. This set of molecules exhibited highly variable catalytic activity. It was an expectation that hammerhead-like molecules would form a subset of the active sequence space. An important goal was therefore to optimise the nucleotide composition between positions 9 and 12 amongst hammerhead-like molecules, within a context of size constraint, and therefore to evolve a linker between A9 and G12 which most efficiently favours equilibration of the active conformation.

SUMMARY OF THE INVENTION

This invention is directed to a selected class of miniribozymes, capable of hybridising with a target RNA to be cleaved, and exhibiting very high cleavage rates at low Mg^{2+} concentration. These miniribozymes may be used both *in vitro* and *in vivo*, and their uses extend to both the diagnostic and therapeutic fields, that is, these miniribozymes may be used as diagnostic or therapeutic agents.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the present invention provides a compound of the formula I:



I

wherein each X represents a nucleotide which may be the same or different and may be substituted or modified in its sugar, base or phosphate; and wherein each $G_{10.1}$ and $C_{11.1}$ each represent a nucleotide which may be substituted or modified in its sugar (which may be ribose or deoxyribose), base or phosphate;

wherein each of C, G, A and U represents a ribonucleotide which may be substituted or modified in its sugar, base or phosphate;

wherein each of $(X)_n$ and $(X)_{n'}$ represents an oligonucleotide having a predetermined sequence which is capable of hybridizing with an RNA target sequence to be cleaved, such RNA target sequence not being present within the compound, and each of n and n' represents an integer which defines the number of nucleotides in the oligonucleotide;

wherein X' represents a ribonucleotide selected from C, G, A and U which may be substituted or modified in its sugar, base or phosphate;

wherein a defines the number of nucleotides in $(X)_a$ and may be 0 or 1 and if 0, the A located 5' of $(X)_a$ is bonded to the G located 3' of $(X)_a$;

wherein each solid line represents a chemical linkage providing covalent bonds between the nucleotides located on either side thereof;

wherein each N represents a nucleotide selected from C, G, A and U/T which may be substituted or modified in its sugar (which may be ribose or deoxyribose), base or phosphate and wherein each H represents a nucleotide selected from C, A and U/T, which may be substituted or modified in its sugar (which may be ribose or deoxyribose), base or phosphate; with the proviso that the sequence 5'-NNHH-3' is not UUUU, CUCC, AAUU or GGCA.

In formula I, C represents a nucleotide in which the base is cytosine, G represents a nucleotide in which the base is guanine, A represents a nucleotide in which the base is adenine, U represents a nucleotide in which the base is uracil, and T represents a nucleotide in which the base is thymine.

In one embodiment of the present invention, the oligonucleotide 3'-$(X)_n$- in the compound of formula I is 3'-$(X)_{n-1}$-A-. More particularly, in this embodiment of the invention the oligonucleotide 3'-$(X)_n$- may be 3'-$(X)_{n-2}$-C-A-.

The compounds of formula I as set out above, include the sequence 5'-NNHH-3' referred to herein as the linker sequence. Preferably, the linker sequence is selected from the following classes of linker sequences:

Class I: YRHH, wherein Y is C or U, R is G or A, and H is C, A or U.

Class II: WYHH, wherein W is A or U, Y is C or U, and H is C, A or U.

Class III: GHHA, wherein H is C, A or U.

Preferred linker sequences in Class I are the sequences CGUU, UGUU and UAAC. Preferred linker sequences in Class II are the sequences ACCC, AUUU, UCCC, AUUC and AUUA. Particularly preferred linker sequences in Class II are the pyrimidine rich subclass of sequences UUHH, wherein H is C, A or U, in particular the sequences UUAC, UUCC, UUUC and UUUA. Preferred linker sequences in Class III are the sequences GUAA and GAUA.

While the preferred linker sequences described above are described as RNA sequences, it will be understood that these preferred linker sequences also include the corresponding DNA sequences.

In general terms, the compounds of the present invention are synthetic, non-naturally occurring oligonucleotide compounds comprising a sequence of nucleotides which includes a catalytic region and hybridizing regions whose sequences are capable of hybridizing with a predetermined RNA target sequence to be cleaved.

The present invention is also directed to compositions comprising a compound of formula I above in association with an acceptable carrier.

The invention is also directed to an oligonucleotide transfer vector containing a nucleotide sequence which on transcription gives rise to a compound of formula I above. The transfer vector may be a bacterial plasmid, a bacteriophage DNA, a cosmid, an eukaryotic viral DNA, a plant DNA virus, a composite geminivirus, a binary plant expression vector (Ri or Ti), an infective phage particle or a portion thereof. The packaged oligonucleotide transfer vector may contain promoter sequences for RNA polymerase II, human $tRNA^{val}$, plant tRNA, human tRNA, snRNA promoter or RNA polymerase III. The invention also includes a host cell transformed by the transfer vector. The host cell may be a prokaryotic host cell or an eukaryotic host cell, such as an E. coli host cell, a monkey COS host cell, a Chinese hamster ovary host cell, a mammalian host cell, a plant host cell, a plant protoplast host cell, a hematopoietic host cell, a stem cell, a hematopoietic progenitor cell, a lymphoid cell, a T-cell, a B-cell, pre-B cell, a CD4+T-cell or a peripheral blood mononuclear cell.

The invention also provides a method of cleaving a target mRNA in a subject which comprises administering to the subject an effective amount of a compound of formula I above or a vector capable of expressing the compound. The administration may be topical in an amount between 1 ng and 10 mg. The administration may also be systemic and administered in an amount between 1 ng and 500 μg/kg weight/day. The administration may also be aerosol administration.

The invention also provides a method of cleaving a target mRNA in a host cell which comprises administering to the host cell an effective amount of a compound of formula I or a vector capable of expressing the compound.

The compound of formula I may further comprise an antisense nucleic acid which is capable of hybridizing with an RNA target sequence. The compound may

further comprise at least one additional non-naturally occurring oligonucleotide compound which comprises nucleotides whose sequence defines a conserved catalytic region and nucleotides whose sequence is capable of hybridizing with a predetermined target sequence. The additional non-naturally occurring oligonucleotide compound may be a hammerhead ribozyme, a minizyme, a hairpin ribozyme, a hepatitis delta ribozyme, an RNAase P ribozyme, a Group I intron, or a combination thereof. See for example: hammerhead ribozyme (Haseloff et al. U.S. Patent No. 5,254,678, issued Oct. 18, 1993; Jennings U.S. Patent No. 5,298,612, issued Mar. 29, 1994); Group I introns, (Cech et al. U.S. Patent No. 4,740,463, issued April 26, 1988; Altman et al. U.S. Patent No. 5,168,053, issued Dec. 1, 1992 or PCT International Publication No WO 92/03566); hepatitis delta ribozymes (PCT International Application No. WO 90/05157) and hairpin ribozymes (European Patent Application No. EP 360,257).

Preferred cleavage sites in the target RNA have the sequence "NUH" (wherein N represents C, G, A or U and H represents C, A or U), preferably GUC, GUU, GUA, UUA and UUC. By way of example, suitable reaction conditions may comprise a temperature from about 4 degree(s) C. to about 60 degree(s) C., preferably from about 10 degree(s) to 45 degree(s) C., more preferably from about 20 degree(s) to 43 degree(s) C.; pH from about 6.0 to about 9.0 and concentration of divalent cation (such as Mg^{2+}) from about 0.1 to about 100 mM, preferably from about 1 to about 100 mM (most preferably 1 to 20 mM). The nucleotides of the sequences $(X)_n$ and $(X)_{n'}$ of the compounds above may be of any number and sequence sufficient to enable hybridization with the nucleotides in the target RNA, as described herein. Ribozymes containing a small number of nucleotides in each of the groups $(X)_n$ and $(X)_{n'}$ of the compounds above (such as four nucleotides) would generally be incubated at lower temperatures, such as about 20 degree(s) C. to about 25 degree(s) C. to aid hybridizing with the nucleotide sequences in the substrate. The number of nucleotides n and n' in $(X)_n$ and $(X)_{n'}$ are not necessarily equal. Preferably, the sum of n+n' is greater than 14.

The invention is also directed to covalently-linked multiple ribozymes, where each ribozyme is directed to a target sequence which may be the same or different. In addition these compounds may be covalently attached to an antisense molecule which may be 10 to 100 bases in length. Antisense sequences capable of hybridizing to an RNA in a mammal or plant are well known see (Shewmaker et al. U.S. Patent No. 5,107,065, issued April 21, 1992). As the ribozyme acts as an enzyme, showing turnover, the ratio of ribozyme to substrate may vary widely.

A target RNA containing a suitable cleavage site such as an NUH site as described above may be incubated with a compound described above. The nucleotide sequences $(X)_n$ and $(X)_{n'}$ of the compounds above are selected to hybridize with their substrate. Preferably, the sequences $(X)_n$ and $(X)_{n'}$ do not naturally occur covalently bound to the sequences 3'-AAG-5' and 5'-CUGA-3', respectively. They may be selected so as to be complementary to nucleotide sequences flanking the cleavage site in the target RNA. On incubation of the ribozyme or ribozyme composition and its substrate, an enzyme/substrate complex is formed as a result of base pairing between corresponding nucleotides in the ribozyme and the substrate. Nucleotide sequences complementary to $(X)_n$ and $(X)_{n'}$ of the compounds above flanking the cleavage site in the substrate may form a double stranded duplex with $(X)_n$ and $(X)_{n'}$ as a result of base pairing, which base pairing is well known in the art (see for example: Sambrook, 1989, Molecular Cloning, A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press). The formation of a double stranded duplex between the nucleotides may be referred to as hybridization (Sambrook, 1989). The extent of hybridization or duplex formation between the ribozyme and its substrate can be readily assessed, for example, by labelling one or both components, such as with a radiolabel, and then subjecting the reaction mixture to polyacrylamide gel electrophoresis under non-denaturing conditions (Sambrook, 1989). If the target is cleaved specifically on incubation with the compound, the compound is active and falls within the scope of this invention. Accordingly, a ribozyme containing substituted or modified nucleotides in the conserved region may be simply tested for endonuclease activity in a routine manner.

As will be readily appreciated by workers in the field to which this invention relates, the cleavage of a target RNA may be readily assessed by various methods well known in the art (see for example: Sambrook, 1989). Cleavage may, for example, be assessed by running the reaction products (where the substrate is radioactively labelled) on acrylamide, agarose, or other gel systems under denaturing conditions, and then subjecting the gel to autoradiography or other analytical technique to detect cleavage fragments (Sambrook, 1989).

The invention is also directed to an oligonucleotide transfer vector containing a nucleotide sequence or sequences which on transcription gives rise to a compound of formula I above. The transfer vector may be a bacterial plasmid, a recombinant bacterial plasmid, a bacteriophage DNA, a cosmid, or an eukaryotic viral DNA. The transfer vector may also contain an appropriate transcription promoter sequence such as that for RNA polymerase II, RNA polymerase III, a viral promoter such as SV40 or HIV LTR, a plant promoter such as CaMV S35 or a promoter associated with animal health. The vector may also contain an appropriate termination sequence. Preferably, the plant or animal promoter is capable of expression in a regulated manner. Such promoter control regions would be regulated by endogenous signals to direct either tissue specific or temporal expression, or by externally administered compounds to elicit transcription of downstream sequences. It may also contain sequences to effect integration into the host genome or episomal replication in the host cell.

The invention also provides a host cell transformed by the transfer vector as mentioned above, which may be a prokaryotic host cell or an eukaryotic host cell e.g. a yeast cell or yeast protoplast, E. coli host cell, a monkey host cell (e.g. COS), a Chinese hamster ovary host cell, a mammalian host cell, a plant host cell, or a plant protoplast host cell.

In one embodiment, there is provided a packaged oligonucleotide transfer vector, as mentioned hereinabove, which is a plant virus, a composite mammalian virus, a geminivirus, a Ti or Ri plasmid, an infective phage particle or portion thereof.

The nucleotides X in the compounds of formula I may be in the form of deoxyribonucleotides, ribonucleotides, deoxyribonucleotide/ribonucleotide hybrids, or derivatives thereof as herein described.

The flanking sequences $(X)_n$ and $(X)_{n'}$ in the compounds of formula I may be chosen to optimize stability of the ribozyme from degradation. For example, deoxyribonucleotides are resistant to the action of ribonucleases. Modified bases, sugars or phosphate linkages of nucleotides, such as phosphoramidate or phosphorothioate linkages in the sugar phosphate chain of $(X)_n$ and $(X)_{n'}$ may also provide resistance to nuclease attack. Binding affinity may also be optimized in particular circumstances, by providing nucleotides solely in the form of ribonucleotides, deoxyribonucleotides, or combinations thereof. In some circumstances it may be necessary to optimize the composition of the sequences $(X)_n$ and $(X)_{n'}$ to maximize target RNA cleavage. The cleavage activity of ribozymes having flanking nucleotide sequences which hybridize to target sequences and which are comprised wholly of deoxyribonucleotides may, in some circumstances, have reduced activity. In such circumstances optimization may involve providing a mixture of deoxyribonucleotides and ribonucleotides in the nucleotide sequences $(X)_n$ and $(X)_{n'}$. For example, nucleotides in the ribozyme which are proximal to the cleavage site in a target RNA may be in the form of ribonucleotides.

The respective 3' and 5' termini of the sequences $(X)_n$ and $(X)_{n'}$ or alternatively the 3' and 5' end termini of the ribozyme, may be modified to stabilize the ribozyme from degradation. For example, blocking groups may be added to prevent terminal nuclease attack, in particular 3'-5' progressive exonuclease activity. By way of example, blocking groups may be selected from substituted or unsubstituted alkyl,

substituted or unsubstituted phenyl, and substituted or unsubstituted alkanoyl groups. Substituents may be selected from C_1 - C_5 alkyl; halogens such as F, Cl or Br; hydroxy; amino; C_1 - C_5 alkoxy and the like. Alternatively, nucleotide analogues such as phosphorothioates, methylphosphonates or phosphoramidates, or nucleoside derivatives (such as alpha - anomer of the ribose moiety) which are resistant to nuclease attack may be employed as terminal blocking groups. The blocking group may be an inverted linkage such as a 3' 3' thymidine linkage or a 5' 5' pyrophosphate linkage as in the guanosine cap.

Alternatively, groups which alter the susceptibility of the ribozyme molecule to nucleases may be inserted into the 3' and/or 5' end of the ribozyme. For example, 9-amino-acridine attached to the ribozyme may act as a terminal blocking group to generate resistance to nuclease attack on the ribozyme molecules and/or as an intercalating agent to aid endonucleolytic activity. It will be readily appreciated that a variety of other chemical groups, e.g. spermine or spermidine, could be used in a related manner.

It is also possible to stabilize the ribozyme from degradation by embedding it in an RNA molecule. These molecules can be produced either *in vitro* or *in vivo* by DNA coding sequences being operably linked to transcriptional control sequences as appropriate. Examples of RNA molecules into which ribozymes could be inserted may include, but are not limited to, tRNA, mRNA, rRNA, snRNA or other RNA molecules. In addition, the ribozyme may be inserted into an engineered stable stem loop structure. The compound may also be coupled with other stabilizing structures such as a transcription terminator on the 3' end such as the T7 terminator, ρ-independent terminator, cry element (Gelfand et al. U.S. Patent No. 4,666,848, issued May 19, 1987) or the TrpE terminator. Furthermore, sequences such as the poly(A) addition signal AATAAA may be added. In addition, strategies involving changing the length of the 3' noncoding region may be used (see Gillies, U.S. Patent No. 5,149,635, issued September 22, 1992). Alternatively, a stabilizing sequence or protein binding domain

(see PCT International application WO 94/10301) may be used. Further, it is possible to insert the compound into a DNA molecule as well.

The compounds of this invention may be covalently or non-covalently associated with affinity agents such as proteins, steroids, hormones, lipids, nucleic acid sequences, intercalating molecules (such as acridine derivatives, for example 9-amino acridine) or the like to modify binding affinity for a substrate nucleotide sequence or increase affinity for target cells, or localization in cellular compartments or the like. For example, the ribozymes of the present invention may be associated with RNA binding peptides or proteins which may assist in bringing the ribozyme into juxtaposition with a target nucleic acid such that hybridization and cleavage of the target sequence may take place. Nucleotide sequences may be added to the respective 3' and 5' termini of the sequences $(X)_n$ and $(X)_{n'}$ or alternatively the 3' and 5' end termini of the ribozyme to increase affinity for substrates. Such additional nucleotide sequences may form triple helices with target sequences which may enable interaction with an intramolecularly folded substrate. Alternatively, modified bases (for example, non-natural or modified bases as described in Saenger, 1984, Principles of Nucleic Acid Structure, Springer-Verlag N.Y.) within the additional nucleotide sequences may be used that will associate with either single stranded or duplex DNA generating base pair, triplet, or quadruplet, interactions with nucleotides in the substrate. Suitable bases would include inosine, 5-methylcytosine, 5-bromouracil and other such bases as are well known in the art, as described, for example, in Saenger, 1984.

The compounds of this invention may be produced by nucleotide synthetic techniques which are known in the art. Generally, such synthetic procedures involve the sequential coupling of activated and protected nucleotide bases to give a protected nucleotide chain, whereafter protecting groups may be removed by suitable treatment. Preferably the compounds will be synthesized on an automated synthesizer such as those made by Applied Biosystems (a Division of Perkin Elmer), Pharmacia or Millipore. Alternatively, the ribozymes in accordance with this invention may be

produced by transcription of nucleotide sequences encoding said ribozymes in host-cells or in cell free systems utilizing enzymes such as T3, SP6 or T7 RNA-polymerase.

In addition to being synthesized chemically, ribozymes with modified nucleotides may be synthesized enzymatically. The phosphodiester bonds of RNA can be replaced by phosphorothioate linkages by *in vitro* transcription using nucleoside α-phosphorothiotriphosphates. T7 RNA polymerase specifically incorporates the Sp isomer of α-phosphorothiotriphosphate with inversion of configuration to produce the Rp isomer of the phosphorothioate linkage. Similarly, T7 RNA polymerase is also able to incorporate 2' O modified nucleotide triphosphates, including 2' O-methyl, 2'-fluoro and 2' amino modified nucleoside triphosphates.

Nucleotides represented in the compounds for formula I above comprise a sugar, base, and a monophosphate group or a phosphodiester linkage. Accordingly, nucleotide derivatives or modifications may be made at the level of the sugar, base, monophosphate groupings or phosphodiester linkages. It is preferred that the nucleotides in the compounds above be ribonucleotides or RNA/DNA hybrids, however, other substitutions or modifications in the nucleotide are possible providing that endonuclease activity is not lost.

In one aspect of this invention, the sugar of the nucleotide may be a ribose or a deoxyribose such that the nucleotide is either a ribonucleotide or a deoxyribonucleotide, respectively. Furthermore, the sugar moiety of the nucleotide may be modified according to well known methods in the art (see for example: Saenger, 1984). This invention embraces various modifications to the sugar moiety of nucleotides as long as such modifications do not abolish cleavage activity of the ribozyme. Examples of modified sugars include replacement of secondary hydroxyl groups with halogen, amino or azido groups; 2'-alkylation; conformational variants such as the 2'-hydroxyl being cis-oriented to the glycosyl C_1 -N link to provide

arabinonucleosides, and conformational isomers at carbon C_1 to give alpha -nucleosides, and the like. In addition, the invention is directed to compounds with a substituted 2'-hydroxyl, such as 2' O-allyl or 2' O-methyl. Alternatively, the carbon backbone of the sugar may be substituted, such as in 2' C-allyl.

Accordingly, the base of the nucleotide may be adenine, 2-amino adenine, cytosine, guanine, hypoxanthine, inosine, methyl cytosine, thymine, xanthine, uracil, or other methylated bases.

Nucleotide bases, deoxynucleotide bases, and ribonucleotide bases are well known in the art and are described, for example in Saenger, (1984). Furthermore, nucleotide, ribonucleotide, and deoxyribonucleotide derivatives, substitutions and/or modifications are well known in the art (see for example: Saenger, 1984); and these may be incorporated in the ribozyme made with the proviso that endonuclease activity of the ribozyme is not lost. As mentioned previously, endoribonuclease activity may be readily and routinely assessed.

In addition, a large number of modified bases are found in nature, and a wide range of modified bases have been synthetically produced (see for example: Saenger, 1984). For example, amino groups and ring nitrogens may be alkylated, such as alkylation of ring nitrogen atoms or carbon atoms such as N_1 and N_7 of guanine and C_5 of cytosine; substitution of keto by thioketo groups; saturation of carbon-carbon double bonds, and introduction of a C-glycosyl link in pseudouridine. Examples of thioketo derivatives are 6-mercaptopurine and 6-mercaptoguanine. Bases may be substituted with various groups, such as halogen, hydroxy, amine, alkyl, azido, nitro, phenyl and the like.

The phosphate moiety of nucleotides or the phosphodiester linkages of oligonucleotides are also subject to derivatization or modifications, which are well known in the art. For example, replacement of oxygen with nitrogen, sulphur or carbon

gives phosphoramidates, phosphorothioates or phosphorodithioates, and phosphonates, respectively. Substitutions of oxygen with nitrogen, sulphur or carbon derivatives may be made in bridging or non bridging positions. It has been well established from work involving antisense oligonucleotides that phosphodiester and phosphorothioate derivatives may efficiently enter cells (particularly when of short length), possibly due to association with a cellular receptor. Methylphosphonates are readily taken up by cells probably by virtue of the electrical neutrality.

A further aspect of the invention provides alternative linkages such as an amide, a sulfonamide, a hydroxylamine, a formacetal, a 3'-thioformacetal, a sulfide, or an ethylene glycol function to replace the conventional phosphodiester linkage. These modifications may increase resistance towards cellular nucleases and/or improve pharmacokinetics.

Detailed information on synthesis of protected nucleotides and their incorporation into modified ribozymes is provided in International Patent Application No. PCT/AU96/00343 (WO 96/40806), the contents of which are incorporated by reference into the present application.

Possible nucleotide modifications include the following, by way of example:

Sugar modifications may be 2' fluoro, 2' amino, 2' O-allyl, 2' C-allyl, 2' O-methyl, 2' O-alkyl, 4'-thio-ribose, α-anomer, arabinose, other sugars, or non-circular analogues.

Phosphate modifications may be phosphorothioate (non-bridging), phosphorodithioate (non-bridging), 3' bridging phosphorothioate, 5' bridging phosphorothioate, phosphoramidate, 3' bridging phosphoramidate, 5' bridging phosphoramidate, methyl phosphonate, other alky/phosphonates or phosphate triesters

The phosphodiester linkage may be replaced by an amide, carbamate, thiocarbamate, urea, amine, hydroxylamine, formacetal, thioformacetal, allyl ether, allyl, ether, or thioether linkage. Alternatively, the phosphodiester linkage and the ribose may be replaced by the amide backbone in a PNA (peptide nucleic acid) linkage.

Base modifications may be purine, 2,6-diaminopurine, 2-aminopurine, 0^6-methylguanosine, 5-alkenylpyrimidine, 5-propyne, inosine, 5-methylcytosine, pseudouridine, abasic (ribose or deoxyribose).

Some nucleotides may be replaced with the following chemical linkers: 1,3-propane-diol, alkane-diols, or various polymers of ethyleneglycol, tetraethylene glycol or hexaethyleneglycol.

Other modifications to the 3' end may be selected from: 3'-3' inverted linkage (inverted nucleotide or inverted abasic nucleotide), 3'-3' linked abasic ribose, or end-capped (methoxyethylamine phosphoramidate).

International Patent Application No. PCT/AU96/00403 sets out in detail methods by which these modified nucleotides may be synthesised, as well as nucleotide modifications which have been tested in ribozymes.

Any combination of the above listed nucleotide modifications, substitutions, or derivatizations, made at the level of the sugar, base, monophosphate groupings or phosphophodiester linkages, may be made in the compounds of the present invention provided that endonuclease activity is not lost.

The compounds of this invention may be incorporated and expressed in cells as a part of a DNA or RNA transfer vector, or a combination thereof, for the maintenance, replication and transcription of the ribozyme sequences of this invention.

Nucleotide sequences encoding the compounds of this invention may be integrated into the genome of a eukaryotic or prokaryotic host cell for subsequent expression (for example as described by Sambrook, 1989). Genomic integration may be facilitated by transfer vectors which integrate into the host genome. Such vectors may include nucleotide sequences, for example of viral or regulatory origin, which facilitate genomic integration. Methods for the insertion of nucleotide sequences into a host genome are described for example in Sambrook et al. (1989).

Nucleic acid sequences encoding the ribozymes of this invention and integrated into the genome of a host cell preferably include promoter and enhancer elements operably linked to the nucleotide sequence encoding the ribozyme of this invention, together with an appropriate termination sequence, so as to be capable of expressing said ribozyme in a eukaryotic (such as animal or plant)or prokaryotic (such as bacteria) host cell. Ideally, the promoter and enhancer elements are designed for expression in a tissue and/or developmentally specific manner.

Additionally, the compounds of the present invention may be prepared by methods known per se in the art for the synthesis of RNA molecules, (for example, according to recommended protocols of Promega, Madison, Wis., USA). In particular, the ribozymes of the invention may be prepared from a corresponding DNA sequence (DNA which on transcription yields a ribozyme, and which may be synthesized according to methods known per se in the art for the synthesis of DNA) operably linked to an RNA polymerase promoter such as a promoter for T3 or T7 polymerase or SP6 RNA polymerase. A DNA sequence corresponding to a ribozyme of the present invention may be ligated into a DNA transfer vector, such as plasmid or bacteriophage DNA. Where the transfer vector contains an RNA polymerase promoter operably linked to DNA corresponding to a ribozyme, the ribozyme may be conveniently produced upon incubation with an RNA polymerase. Ribozymes may, therefore, be produced *in vitro* by incubation of RNA polymerase with an RNA polymerase promoter operably linked to DNA encoding a ribozyme, in the presence of ribonucleotides and an

appropriate buffer. *In vivo*, prokaryotic or eukaryotic cells (including mammalian, plant and yeast cells) may be transfected with an appropriate transfer vector containing genetic material encoding a ribozyme in accordance with the present invention, operably linked to an RNA polymerase promoter such that the ribozyme is transcribed in the host cell. Transfer vectors may be bacterial plasmids or viral (RNA and DNA) or portion thereof. Nucleotide sequences corresponding to ribozymes are generally placed under the control of strong promoters such as the lac, SV40 late, SV40 early, metallothionein, or lambda promoters. Particularly useful are promoters regulated in a tissue or a temporal (developmental) specific manner, or a tightly regulated inducible promoter suitable for gene therapy, which may be under the control of exogenous chemicals. The vector may be an adenovirus or an adeno-associated virus (see for example PCT International Publication No. WO 93/03769, "Adenovirus Mediated Transfer of Genes to the Gastrointestinal Tract", PCT International Publication No. WO 94/11506, "Adenovirus-Mediated Gene Transfer to Cardiac and Vascular Smooth Muscle," PCT International Publication No. WO 94/11522, "Vector for the Expression of Therapy-Relevant Genes," PCT International Publication No. WO 94/11524, "Targetable Vector Particles," PCT International Publication No. WO 94/17832, "Targeting and Delivery of Genes and Antiviral Agents into Cells by the Adenovirus Penton"). Ribozymes may be directly transcribed *in vivo* from a transfer vector, or alternatively, may be transcribed as part of a larger RNA molecule. For example, DNA corresponding to ribozyme sequences may be ligated into the 3' end of a reporter gene, for example, after a translation stop signal. Larger RNA molecules may help to stabilize the ribozyme molecules against nuclease digestion within cells. On translation, the reporter gene may give rise to a protein, possibly an enzyme whose presence can be directly assayed.

The compounds of this invention may be involved in gene therapy techniques where, for example, cells from a human suffering from a disease, such as HIV, are removed from a patient, treated with the ribozyme or transfer vector encoding the ribozyme to inactivate the infectious agent, and then returned to the patient to

repopulate a target site with resistant cells, so called *ex vivo* therapy. Such cells would be resistant to HIV infection and the progeny thereof would also confer such resistance. In the case of HIV, nucleotide sequences encoding ribozymes of this invention capable of inactivating the HIV virus may be integrated into the genome of lymphocytes or may be expressed by a non-integrating vector such as adenovirus. Alternatively, the nucleotide sequences may be expressed by a retrovirus or modified retrovirus known for use in the treatment of HIV.

A transfer vector such as a bacterial plasmid or viral RNA or DNA or portion thereof, encoding one or more of the compounds of this invention, may be transfected into cells of an organism *in vivo*. Once inside the cell, the transfer vector in some cases may replicate and be transcribed by cellular polymerases to produce ribozyme RNAs which may have ribozyme sequences of this invention; the ribozyme RNAs produced may then inactivate a desired target RNA. Alternatively, a transfer vector containing one or more ribozyme sequences may be transfected into cells by electroporation, PEG, high velocity particle bombardment or lipofectants, or introduced into cells by way of micromanipulation techniques such as microinjection, such that the transfer vector or a part thereof becomes integrated into the genome of the host cell. Transcription of the integrated genetic material gives rise to ribozymes, which act to inactivate a desired target RNA. Transfer vectors expressing ribozymes of this invention may be capable of replication in a host cell for stable expression of ribozyme sequences. Alternatively, transfer vectors encoding ribozyme sequences of this invention may be incapable of replication in host cells, and thus may result in transient expression of ribozyme sequences. Methods for the production of DNA and RNA transfer vectors, such as plasmids and viral constructs are well known in the art and are described for example by Sambrook et al. (1989).

Transfer vectors would generally comprise the nucleotide sequence encoding the ribozyme of this invention, operably linked to a promoter and other regulatory sequences required for expression and optionally replication in prokaryotic and/or

eukaryotic cells. Suitable promoters and regulatory sequences for transfer vector maintenance and expression in plant, animal, bacterial, and other cell types are well known in the art.

The ribozymes of the present invention have extensive therapeutic and biological applications. For example, disease-causing viruses in man and animals may be inactivated by administering to a subject infected with a virus, a ribozyme in accordance with the present invention adapted to hybridize to and cleave specific RNA transcripts of the virus. Such ribozymes may be delivered by parenteral or other means of administration. Alternatively, a subject infected with a disease causing virus may be administered a non-virulent virus such as vaccinia or adenovirus which has been genetically engineered to contain DNA corresponding to a ribozyme operably linked to an RNA promoter, such that the ribozyme is transcribed in the cells of the host animal, transfected with the engineered virus, to effect cleavage and/or inactivation of the target RNA transcript of the disease causing virus.

The ribozymes of the present invention have particular application to viral diseases caused for example, by the herpes simplex virus (HSV) or the AIDS virus (HIV). Further examples of human and animal disease which may be treated with the ribozymes of this invention include psoriasis, cervical preneoplasia, papilloma disease, bacterial and prokaryotic infection, viral infection and neoplastic conditions associated with the production of aberrant RNAs such as occurs in chronic myeloid leukemia. Diseases or infections which may be treated in plants with ribozymes of this invention include fungal infection, bacterial infections (such as Crown-Gall disease) and disease associated with plant viral infection. Of particular interest would be compounds targeting genes associated with male gametophyte development, (examples include PCT International Publication No. WO 92/18625, entitled "Male-Sterile Plants, Method For Obtaining Male-Sterile Plants And Recombinant DNA For Use Therein"; U.S. Patent No. 5,254,802, entitled "Male Sterile Brassica Plants ";PCT International Publication No. WO 93/25695, entitled "Maintenance of Male-Sterile Plants ";PCT

International Publication No. WO 94/25593, entitled "Method For Obtaining Male-Sterile Plants; PCT International Publication No. WO 94/29465, entitled "Process For Generating Male Sterile Plants").

The period of treatment would depend on the particular disease being treated and could be readily determined by a physician or by a plant biologist as appropriate. Generally treatment would continue until the disease being treated was ameliorated.

The ribozymes of the present invention also have particular application to the inactivation of RNA transcripts in bacteria and other prokaryotic cells, plants, animals and yeast cells. In bacteria, RNA transcripts of, for example, bacteriophage (which cause bacterial cell death) may be inactivated by transfecting a cell with a DNA transfer vector which is capable of producing a ribozyme in accordance with the present invention which inactivates the phage RNA. Alternatively, the ribozyme itself may be added to and taken up by the bacterial cell to effect cleavage of the phage RNA. Similarly, eukaryotic and prokaryotic cells in culture may, for example, be protected from infection or disease associated with mycoplasma infection, phage infection, fungal infection and the like.

RNA transcripts in plants may be inactivated using ribozymes encoded by a transfer vector such as the Ti plasmid of *Agrobacterium tumefaciens*. When such vectors are transfected into a plant cell and integrated, the ribozymes are produced under the action of RNA polymerase and may effect cleavage of a specific target RNA sequence. Endogenous gene transcripts in plant, animal or other cell types may be inactivated using the compounds of the present invention. Accordingly, undesirable phenotypes or characteristics may be modulated. It may, for example, be possible using the ribozymes of the present invention to remove stones from fruit or treat diseases in humans which are caused by the production of a deleterious protein, or over-production of a particular protein. The compounds described above may be used to effect male sterility by destroying the pollen production in a plant. Furthermore, for

the *in vivo* applications of the ribozymes of this invention in humans, animals, plants, and eukaryotic and prokaryotic cells, such as in phenotypic modification and the treatment of disease, it is necessary to introduce the ribozyme into cells whereafter, cleavage of target RNAs takes place. In vivo applications are highly suitable to the compounds as discussed herein. Methods for the introduction of RNA and DNA sequences into cells, and the expression of the same in prokaryotic and eukaryotic cells are well known in the art. The same widely known methods may be utilized in the present invention.

The compounds of this invention may be incorporated into cells by direct cellular uptake, where the ribozymes of this invention would cross the cell membrane or cell wall from the extracellular environment. Agents may be employed to enhance cellular uptake, such as liposomes or lipophilic vehicles, cell permeability agents, such as dimethylsulfoxide, and the like.

The compounds of the present invention may be combined with pharmaceutically and veterinarally acceptable carriers and excipients which are well known in the art, and include carriers such as water, saline, dextrose and various sugar solutions, fatty acids, liposomes, oils, skin penetrating agents, gel forming agents and the like, as described for example in Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Co., Easton, Pa., which is incorporated herein by reference.

Agriculturally acceptable carriers and excipients are well known in the art and include water; surfactants; detergents; particularly biodegradable detergents; talc; inorganic and/or organic nutrient solutions; mineral earths and clays; calcium carbonate; gypsum; calcium sulfate; fertilizers such as ammonium sulfate, ammonium phosphate, urea, carborundum, and *Agrobacterium tumefaciens*; and natural products of vegetable origin such as, for example, grain, meals and flours, bark meals; and the like.

The compounds of this invention may be provided in a composition with one or more anti-viral, anti-fungal, anti-bacterial, anti-parasitic, anti-protozoan or anthelminthic agents, herbicides, pesticides or the like, for example as described in the Merck Index (1989) 11th Edition, Merck & Co. Inc.

By way of example only, therapeutic compositions of this invention may be directed against Herpes Simplex virus types 1 and 2, psoriasis, cervical preneoplasia, papilloma disease, and bacterial and prokaryotic infection. Such treatments may, for example, involve topical application of ribozyme to the site of disease. For example, in the treatment of Herpes virus lesions, ribozymes may be formulated into a cream containing a concentration of 0.1 nM to 100 mM ribozyme, preferably 1 nM to 1 mM. The cream may then be applied to the site of infection over a 1 to 14 day period in order to cause amelioration of symptoms of the infection. Prior to the final development of topical formulations for the treatment of virus infection, effectiveness and toxicity of the ribozymes and formulations involving them may, for example, be tested on an animal model, such as scarified mouse ear, to which virus particles, such as 2×10^6 plaque forming units are added. A titer of infectious virus particles in the ear after treatment can then be determined to investigate effectiveness of treatment, amount of ribozyme required and like considerations. Similar investigations in animal models prior to human trials may also be conducted, for example, in respect of the treatment of psoriasis, papilloma disease, cervical preneoplasia, and in diseases such as HIV infection, bacterial or prokaryotic infection, viral infection and various neoplastic conditions which involve a deleterious RNA species.

Compositions for topical application are generally in the form of creams, where the ribozymes of this invention may be mixed with viscous components. The compounds of this invention may be incorporated into liposomes or other barrier type preparations to shield the ribozymes from nuclease attack or other degradative agents (such as nucleases and adverse environmental conditions such as UV light).

Compositions may be provided as unit dosages, such as capsules (for example gelatin capsules), tablets, suppositories and the like. Injectable compositions may be in the form of sterile solutions of ribozyme in saline, dextrose or other media. Compositions for oral administration may be in the form of suspensions, solutions, syrups, capsules, tablets and the like. Ribozymes may also be provided in the form of an article for sustained release, impregnated bandages, patches and the like. The compounds of this invention may be embedded in liposomes or biodegradable polymers such as polylactic acid. Pharmaceutical compositions which may be used in this invention are described, for example, in Remington's Pharmaceutical Sciences, see above.

The present invention is further directed to a plant DNA expression cassette comprising a gene sequence flanked by promoter and terminator sequences at its 5' and 3' ends respectively wherein said genetic sequence on expression provides a ribozyme RNA. The DNA cassette may further be part of a DNA transfer vector suitable for transferring the DNA cassette into a plant cell and insertion into a plant genome. In a most preferred embodiment of the present invention, the DNA cassette is carried by broad host range plasmid which is capable of transformation into plant cells using *Agrobacterium* comprising Ti DNA on the left and right borders, a selectable marker for prokaryotes, a selectable marker for eukaryotes, a bacterial origin of replication and optional plant promoters and terminators. The present invention also includes other means of transfer such as genetic bullets (e.g. DNA-coated tungsten particles, high-velocity micro projectile bombardment) and electroporation amongst others.

The present invention is also directed to a transgenic plant resistant to a virus, its genome containing a sequence which gives rise, on transcription, to the nucleic acid molecule mentioned above. This transgenic plant, including fruits, and seeds thereof, may be from alfalfa, apple, arabidopsis, barley, bean, canola (oilseed rape), cantaloupe, carnation, cassava, casuarina, clover, corn, cotton, courgette, cucumber,

eucalyptus, grape, melon, papaya, pepper, potato, rice, rose, snap dragon, soybean, squash, strawberry, sunflower, sweet pepper, tobacco, tomato, walnut, wheat or zucchini. Also included are the plant cells transformed by the above-mentioned transfer vector comprising a nucleotide sequence which is, or on transcription gives rise to, the nucleic acid molecule.

Throughout this specification, unless the context requires otherwise, the word "comprise", and or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Further features of the present invention are more fully described in the following Example(s). It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the present invention, and should not be understood in any way as a restriction on the broad description of the invention as set out above.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. N18g0 RNA plus substrate showing construction of the size constrained random (N = 18) domain. The diversity of g0 was 4^{18} (6.9×10^{10}). The design is a bimolecular configuration analogous to that proposed by Haseloff and Gerlach (13), comprising two antisense regions separated by a metal dependent catalytic domain. Helix I and III were formed by association with a biotinylated RNA substrate (IL2bioS). Helix III is not shown in full. Notation on the random positions indicates correspondent nucleotide base identity and positions for the hammerhead (53). In the hammerhead, helix II is an extended RNA hairpin comprising 3 - 6 Watson-Crick base pairs. The enzyme strand (E) promotes 2'-O^- mediated cleavage of the substrate strand (S) immediately 3' to C17 (indicated by arrow).

Figure 2. (a) Scheme for *in vitro* selection. The transcribed random pool RNA was directed in *trans* against a 29mer synthetic RNA substrate (IL2bioS) comprising a 13 nt segment of human IL2 mRNA. Annealed duplexes were immobilised to an MPG-avidin solid phase via a 3' substrate biotin. Unbound material was removed and active structures were eluted by adding $MgCl_2$. Molecules capable of supporting Mg^{2+} dependent cleavage became selectively disassociated from the solid phase due to reduced helical stability (ie. tm helix I < tm helix I + III). (b) Shows IL2bioS (substrate) and random (N18) RNA, in *trans*. (c) Shows the structure of the N4 random population. The selected fraction was amplified (RT-PCR) using primers P1/P2 (N18) and P1/P3 (N4). P2 and P3 encoded T7 promoters, enabling transcription of the subsequent generation.

Figure 3. Enrichment of Mg^{2+} dependent phosphodiesterase activity in the N18 pool RNA using a non-stringent high $MgCl_2$ (20 mM) regime. The activity of separate RNA pools generated by iterative *in vitro* selection was assayed in conditions of substrate excess. Cleavage assay conditions were; ribozyme 100 nM, substrate (IL2S15 15mer RNA) 40 μM, 10 mM $MgCl_2$, pH 8.0, 37°C. Cleavage activity (k_{cat}) displayed by the random pool RNA became detectable after 3 rounds of selection (g3), and peaked in g4, ie. further enrichment of the k_{cat} phenotype could not be obtained.

Figure 4. Sequence alignment showing composition of the N18 domain in the selected populations. Variable regions are highlighted. (a) Shows a sampling of the cloned fraction of the g4 population and indicates the state of enrichment after 4 rounds of low stringency selection. Linker positions are numbered 10.1, L.1 - L.4, and 11.1. (b) The sampled fraction of the g6 populations showing the impact of high stringency selection on the identity of the variable (linker) region (positions 10.1 - 11.1 inclusive).

Figure 5. $MgCl_2$ concentration and incubation time used during selection, showing (a) the inception and differences between the three high stringency lineages established from the N18g4 pool, and (b) the protocol used for the N4 experiment.

Figure 6. Sequence data acquired from the N4g3 population. All sequences correspond to the derivation; 5' NNHH (linker positions L.1 - L.4). Three classes of molecule can be identified; YRHH, WYHH, GHHA (Y = C/U; R = G/A; W = A/U; H = A/U/C). Numbers after sequences indicate the incidence of that particular motif in N4g3. A highly represented pyrimidine rich subset (UUHH) of class II was identified.

EXAMPLE

In vitro selection was used to enrich for highly efficient RNA phosphodiesterases within a size constrained (18 nt) ribonucleotide domain. The starting population (g0) was directed in *trans* against an RNA oligonucleotide substrate immobilised to an avidin-magnetic phase. 4 rounds of selection were conducted using 20 mM Mg^{2+} to fractionate the population on the basis of divalent metal ion dependent phosphodiesterase activity. The resulting generation 4 (g4) RNA was then directed through a further 2 rounds of selection using low concentrations of Mg^{2+}. Generation 6 (g6) was composed of sets of active, *trans* cleaving minimised ribozymes, containing recognised hammerhead motifs in the conserved nucleotides, but with highly variable linker domains (loop II - L.1-L.4). Cleavage rate constants in the g6 population ranged from 0.004-1.4 min^{-1} at 1 mM Mg^{2+} (pH 8.0, 37°C). Selection was further used to define conserved positions between G(10.1) and C(11.1) required for high cleavage activity at low Mg^{2+} concentration. The kinetic phenotype of these molecules was superior to a hammerhead ribozyme with 4 bp in helix II. At low Mg^{2+} concentration, the disparity in cleavage rate constants increases in favour of the minimised ribozymes. Favourable kinetic traits appeared to be a general property for specific selected linker sequences, as the observation of high rates of catalysis were transferable to a different substrate system.

Materials and methods

Oligonucleotides Oligonucleotides were synthesised on an Applied Biosystems model 394 DNA synthesiser. DNA Phosphoramidite monomers were supplied by PE-Applied Biosystems. RNA phosphoramidites were from Glen Research (Sterling, Virginia). Deprotection and purification was as described previously (25,27). Gel purified oligonucleotides were resuspended in sterile, autoclaved H_2O. Concentrations were determined by UV spectroscopy and samples were stored at -20°C. Zero generation (g0) RNA was transcribed *in vitro* from a synthetic DNA template (N18g0T) containing a T3 promoter sequence. (lower case = deoxyribonucleotide; upper case = ribonucleotide). N18g0T 65mer DNA; 5' ctc ggt acc gtt gat cct (n18) ttg cat tgg gcc ttt agt gag ggt taa tt, (minus strand) T3 promoter underlined. RNA substrate (IL2bioS) was produced directly by solid phase synthesis. Biotin was incorporated using 3' BioTEG phosphoramidite (12 carbon linker) supplied by Auspep (Melbourne, Australia). IL2bioS 29mer RNA; 5' CUC GGU ACC GUU GAU CCU GUC UUG CAU AA-biotin 3' (putative cleavage triplet underlined). N4g0T 49mer 5' ctc ggt acc gtt gat cct gtt tcg (n4) ctc atc agt tgc att ggg ccc tat agt gag tcg tat ta, (minus strand) T7 promoter underlined. Primers used were; T3 15mer 5' aat taa ccc tca cta; P1 17mer 5' ctc ggt acc gtt gat cc; P2 38mer 5' gag gga tcc taa tac gac tca cta tag gcc caa tgc aa; P3 40mer 5' gag gga tcc taa tac gac tca cta tag ggc cca atg caac, T7 promoters (plus strand) underlined. A number of substrates, plus ribozymes with full length helix II, and ribozymes with evolved linkers, were produced by solid phase synthesis to test the comparative efficacy of the evolved miniribozymes versus conventional hammerhead ribozymes in different substrate backgrounds. These were; KrS17 5' UUG CGA GUC CAC ACU Gg (17mer substrate); IL2S19 5' AAC UCC UGU CUU GCA UUGc (19mer substrate); IL2S15 UCC UGU CUU GCA UUg (15mer substrate); KrMc10 5' UCC AGU GUG CUG AUG AGG UAA CGA AAC UCG CAAa (34mer miniribozyme); KrRz 5' CUC CAG UGU GCU GAU GAG UCC UUU UGG ACG AAA CUC GCA AAt (42mer ribozyme); IL2Mc10 5' GCA AUG CAA CUG AUG AGG UAA

CGA AAC AGG AGUt (34mer miniribozyme); IL2Rz 5' GCA AUG CAA CUG AUG AGU CCU UUU GGA CGA AAC AGG AGUt (40mer ribozyme).

Transcription An optimal transcription reaction contained; 0.3-1.0 µM template, 40 mM Tris-HCl pH 8.0, 12-25 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 50 µg/µl BSA, 0.01% v/v triton -X 100, 5 mM of each NTP (Boehringer Mannheim), and 20 U/µl T3 RNA polymerase (Promega) or 10 U/µl T7 RNA polymerase (New England Biolabs). Reactions were incubated at 37°C. To generate labelled (^{32}P) sample, UTP was reduced to 0.4 mM, and $\alpha^{32}P$ UTP (DuPont) was added to 1.0 µCi/ µl. N18g0 templates were prepared for transcription by annealing 65 pmoles N18g0T (65mer) with an excess (75 pmoles) of T3 primer (15mer) and extending with 2 units of Klenow enzyme (Boehringer Mannheim) using standard conditions, followed by phenol/chloroform extraction and ethanol precipitation. N4g0 template was prepared by annealing 400 pmoles of N4g0T with 800 pmoles of P3. Subsequent generation RNA was transcribed from PCR products with primer incorporated T7 promoters (P2 for N18, and P3 for N4). All transcribed ribozymes were purified by Sephadex G-50 (Pharmacia) chromatography, followed by phenol/chloroform extraction and ethanol precipitation. Purified RNA was dissolved in H_2O and stored at -20°C.

N18 selection Pool RNA (50 pmoles) and IL2bioS substrate (25 pmoles) were annealed in 1 mM EDTA / 50 mM Tris-HCl (pH 8.0, 37°C) by heating to 85°C for 2 minutes and cooling slowly to room temperature (to promote duplex formation). The annealing reaction was incubated in 100 µl 1 M NaCl / 1 mM EDTA / 50 mM Tris-HCl (pH 8.0 37°C) for 15 minutes at room temperature with 200 µg avidin paramagnetic porous glass (MPG-Avidin) CPG Inc. (Lincoln Park, New Jersey). Excess ribozyme was used to prevent the binding of free substrate. The solid magnetic phase was captured and the supernatant containing the unbound material was removed. Bound material was equilibrated using 3 washes at 40°C and 3 washes at 25°C (80 µl 1 M NaCl / 1 mM EDTA / 50 mM Tris-HCl, pH 8.0). This was followed by a 15 minute incubation in 80 µl 1 M NaCl / 1 mM EDTA / 50 mM Tris-HCl (pH 8.0) at 37°C.

Supernatant from this treatment provided a negative control (negative selection) which was used to establish a base line for calculating the noise/signal ratio (background/cleavage product) in the selected fraction. Positive selection was initiated by resuspending the solid phase in 80 μl 1 M NaCl / 50 mM Tris-HCl (pH 8.0, 37°C), supplemented with 20 mM $MgCl_2$ (g0 - g4), or 1 - 4 mM $MgCl_2$ (g5 - g6). Incubation in the selection buffer was for 15 minutes (g0 - g4), or 1 minute (g5 - g6).

N4 selection N4 pool RNA (300 pmoles) and IL2bioS (150 pmoles) were annealed in 50 mM Tris-HCl / 1 mM EDTA, pH 7.6, and bound to 400 μg MPG-Avidin. The selection protocol was as described for N18 above. High concentrations of pool RNA and IL2bioS were used to eliminate the effect of possible contamination from N18g4 or g6. Binding and wash buffer was 100 mM NaCl / 100 mM KCl / 50 mM Tris-HCl / 0.2 mM EDTA, pH 7.6. Selection buffer was 1 mM $MgCl_2$ / 100 mM NaCl / 100 mM KCl / 50 mM Tris-HCl, pH 7.6. Three rounds of selection were conducted, with 1 minute incubation at 37°C.

Amplification and cloning The supernatant representing selected RNA was ethanol precipitated in the presence of a glycogen carrier (Sigma). Precipitated RNA was dissolved in 10 μl H_2O and reverse transcribed with a large excess of P1 (100 pmoles). Reverse transcription (RT) reactions contained 1 U/μl AMV reverse transcriptase (Boehringer Mannheim), 1 x RT buffer (Boehringer Mannheim), 2 mM of each dNTP (Promega), and 0.5 U/ μl RNAsin (Promega) in a 20 μl final volume. The RT reaction was incubated for 15 minutes at 37°C. 0.5 μl of the RT product was used directly for PCR. Thermal cycling was performed using a Corbett Research FTS-1 thermal sequencer (Corbett Industries, Sydney). Reactions contained 0.01 U/μl Taq DNA polymerase (Boehringer Mannheim), 1 x Taq reaction buffer (Boehringer Mannheim), 1.5 mM $MgCl_2$, 0.25 mM each dNTP, 0.625 μM primers (P1 and P2). Parameters for thermal cycling were 1 x (94°C, 45 seconds; 45°C, 30 seconds; 72°C, 2 minutes), 4 - 20 x (94°C, 30 seconds; 55°C, 30 seconds; 72°C, 2 minutes). The number of cycles required was inversely proportional to the quantity of starting

material, i.e. less cycles were required as the quantity of starting template increased due to ensuing rounds of selection and enrichment of the active component. P2 (N18) and P3 (N4) engineered T7 promoters in the PCR product enabling subsequent transcription as described above. PCR products encoding g4, g6b, g6c, g6d, and N4g1, N4g2 and N4g3, were cloned separately into pBluescript SK+ (Stratagene) using BamHI and KpnI restriction sites. Sequence data was obtained by dideoxy sequencing using Sequenase version 2.0 (United States Biochemical). Cloned material from g4 and g6 was transcribed from pBluescript SK+ linearised with Acc65 I for kinetic assays of individual molecules.

Substrate excess kinetics Kinetic assays were conducted on pooled transcripts from each generation to determine changes in cleavage efficiency. Measurements were made with substrate in excess. Conditions were; 10 nM ribozyme, 4 μM of ^{32}P labelled 15mer IL2 RNA substrate (IL2S15), 50 mMTris-HCl (pH 8.0, 37°C). Reactions were in 25 μl volumes and were initiated by adding $MgCl_2$ (final concentration 10 mM). Samples (2 μl) were taken at 10 minute intervals and quenched immediately in 4 μl of stop solution (95% formamide, 20 mM EDTA, 0.025% tracking dyes). Samples were separated in 15% polyacrylamide/7M urea (1 x TBE) and the amount of cleavage quantified using ImageQuant (Molecular Dynamics). The V_{max} was obtained from the slope of a linear plot of product formation versus time, and converted to k_{cat}; where $k_{cat} = V_{max}/[Rz]$.

Ribozyme excess kinetics Cleavage rates were also measured for individual molecules from the cloned samples using ribozyme in excess of the substrate. The ribozyme was used at 100 nM, and substrate was 5 nM of ^{32}P labelled IL2bioS 29mer RNA. In early experiments the ribozyme concentration was varied to confirm saturating conditions. Reactions were at 37°C in 25 μl 50 mM Tris-HCl (pH 8.0 or pH 7.6, as indicated), and were initiated by the addition of $MgCl_2$ to a final concentration of 10 mM or 1 mM , as indicated. Samples (2 μl) were removed at 0, 20, 40, 60, 120, 300, 600 seconds, and quenched immediately in 4 μl of stop solution (95% of formamide, 20mM

EDTA, 0.01% tracking dyes). Samples were separated in 15% polyacrylamide/7M urea (1 x TBE) and the amount of cleavage quantified using ImageQuant (Molecular Dynamics). Rate constants (k_1) for single-phase reactions were derived by fitting the data to the equation; $P_t = P\infty - (\exp(-k_1t)P_\Delta)$, where P_t is the amount of product at time = t, $P\infty$ is the amount of product at time = ∞, k_1 is the first order rate constant for the reaction, and P_Δ is the difference between the percentage of product at t = ∞ and t = 0. For biphasic reactions, the data was fitted to a double-exponential equation; $P_t = [P_1 - (\exp(-k_1t)P_{\Delta1})] + [P_2 - (\exp(-k_2t)P_2)]$ where k_1 is the rate constant for the first phase, k_2 is the rate constant for the second phase, P_1 is the extent of the first phase, $P_{\Delta1}$ is the difference between the percentage of product at P_1 and t = 0, and P_2 is the extent of the second phase of the reaction.

Results and discussion

The central feature of the selection scheme depicted in Figure 2 is that Mg^{2+} dependent cleavage results in the formation of a ribozyme and 5' cleavage product complex which can be recovered by selective denaturation. The duplex formed between the 3' biotinylated substrate (IL2bioS) and the generation 0 (g0) molecules had an 18 bp double helix 5' of the targeted cleavage site (helix III), and a 6 bp double helix to the 3' of this site (helix I). Using thermodynamic parameters for helix initiation and propagation, provided by Freier et al (34), the t_m for the release of active species carrying the 5' portion of a cleaved substrate was predicted to be ~40°C. The predicted t_m for any given member of the randomised population hybridised to an uncleaved substrate was greater than 90°C. The difference in t_m provided a strong basis of selection for molecules showing *trans* Mg^{2+} dependent phosphodiesterase activity at, or near, the targeted GUC site in the substrate, allowing quantitative recovery of active species. RNA released from the avidin solid phase, into the supernatant, was collected by ethanol precipitation, amplified (RT-PCR), and transcribed for reiterative enrichment (see Methods section).

Selection at 20 mM Mg^{2+}: generation 4 (N18g4) The structure of the starting population (g0) is depicted in Figure 1. Starting from g0 RNA, 5 rounds of selection using 20 mM $MgCl_2$ for 15 minutes at 37°C were performed. The populations of RNA resulting from each round of selection were tested for their ability to cleave a radiolabelled RNA substrate *in vitro*. Generation 3 was the first to show detectable levels of cleavage activity. This activity was improved in generation 4 (g4), but was not increased by additional selection at 20 mM $MgCl_2$, ie. the k_{cat} phenotype of g4 was identical to g5a (Figure 3).

Amplified material from g4 was cloned and sequenced, revealing a complex population (Figure 4a). Nonetheless, it was overwhelmingly composed of molecules sharing conserved nucleotides with the hammerhead ribozyme. Molecules exhibiting the conserved hammerhead-like motifs showed high variability between positions 10.1 and 11.1 (inclusive) and at position 7 (although this last position showed a strong pyrimidine bias). The upper limit of diversity for a population which varies randomly in the linker region (10.1 - 11.1) is 4^6. The random distribution (0.25) of identities at these positions in g4 was compromised to some extent by a high occurrence of U at position 11.1 and A at position L.3. There is also a lower than expected occurrence of G at positions 10.1, L.2, L.3, and 11.1. However, this effect was slight. The same sequence was not sampled more than once, and the distribution of nucleotides in the linker is significantly random. It was therefore reasonable to maintain that the diversity of g4 approached a substantial part of the 4^6 maximum (with some additional variation being contributed by position 7). Presumably this diversity reflected the lack of highly stringent demand on the phenotype.

Several molecules which deviated from the hammerhead design were sequenced. No detectable cleavage activity was observed for any of these molecules when tested independently, nor did they exhibit any pooled activity (results not shown). The selection was conducted in *trans* allowing the possibility of isolating molecules exhibiting dimer dependent activity. Non-hammerhead sequences sampled, could

therefore plausibly represent truncations of potentially active structures whose additionally required components have not been observed in the cloned and sequenced sample. Alternatively, these may represent inactive molecules released due to the equilibrium position of the *trans* RNA duplex. Notable also in g4 is molecule 4.31 (see Figure 4a) which incorporates an extraneous U, presumably as a bulge in helix III. An additional nucleotide can be incorporated between positions 11.1 and 12 (35), and also between 10.1 and 9 (36). In these instances, the additional nucleotide perturbs the structure of helix II, causing reduction, but not loss of activity. In our study, molecule 4.31 exhibited moderate activity at 1 mM Mg^{2+} (Table 1), indicating that a bulge can be accepted here. Whilst 4.31 did not exhibit a high cleavage rate constant, it is not known whether this is attributable to the extraneous nucleotide, or the linker identity (10.1 - 11.1). Molecule 4.31 and several others have only 5 nucleotides in the linker, and generally these cleaved poorly.

Selection at low Mg^{2+} concentrations: generation 6 (N18g6) To select populations of molecules displaying further enhanced kinetic properties, three high stringency lineages were established from g4 RNA, employing shorter cleavage times and reduced Mg^{2+} concentration (Figure 5a), producing g6b, g6c, and g6d. These populations showed rate constants (k_1) significantly higher than those obtained for g4 (data not shown). Cloning and sequencing amplified material from the generation 6 populations revealed that they were much less diverse than N18g4. The reduced diversity was largely accounted for by a significant increase in the frequency of species carrying the sequence 5' G(10.1)NNNNC(11.1) in the linker region. In g4 the occurrence of 5' G(10.1)NNNNC(11.1) was 0.07, ie. close to the expected random incidence (0.25 x 0.25 = 0.0625). The occurrence of GNNNNC in the g6 populations was 0.7 (69.6%), representing a 10-fold enrichment. Of the 69.6% of sequences exhibiting the identity GNNNNC, 96.9% are GNNHHC. This identity is concentrated in g6c and g6d families, ie. those populations evolved under the most stringent conditions of Mg^{2+} supply.

N18g6 ribozymes exhibit a variable tolerance to low Mg^{2+} concentrations

Cleavage kinetics at 1 mM $MgCl_2$, pH 8.0, 37°C with ribozyme in excess, were obtained for 28 molecules (24 of which were g6 individuals). Measurements were also made at 10 mM $MgCl_2$, pH 8.0, 37°C for a subset of 14 molecules (Table 1). The reactions were distinctly biphasic for most molecules both at 1 mM and at 10 mM Mg^{2+}. The activity of different molecules could clearly be delineated by rate constants observed for the first rapid phase. While most molecules exhibited a more uniform slower second phase, where k_2 was in the range 0.05 (0.02 min-1 (data not shown), the extent (P_1) and rate constant (k_1) of the first phase was highly variable. This variation in rate constants for a set of molecules which differed only in the nucleotide composition of the linker between positions A9 and G12, suggested an important role for the linker in structural and catalytic outcomes, where these requirements are only fully satisfied by some, and not other, linker identities. For some molecules, very high rate constants were observed during the first phase of the reaction at both 10 mM and 1 mM Mg^{2+} (see Table 1). It is possible that the linker exerts influence on the equilibrium position between variably active conformations, and that some linkers can cause a redistribution in favour of a more active conformation resulting in high k_1 and P_1. The rate constants and the extents of the first phase were substantially reduced when Mg^{2+} was lowered from 10 mM to 1 mM. However, the reduction in cleavage activity was not uniform. For some molecules k_1 dropped dramatically (20-50 fold). In several instances this reduction involved complete extinguishment of the biphasic character of the reaction. At 1 mM these molecules exhibited slow monophasic kinetics. Other molecules experienced only a moderate reduction in extent and rate of the initial cleavage reaction (ie. in the range of 2-5 fold) when Mg^{2+} was lowered to 1 mM. A subset of linkers could therefore be identified which imparted tolerance to low concentrations of Mg^{2+} (shown above the dotted line in Table 1). This group was distinguished on the basis of exhibiting $k_1 \geq 0.5$ min^{-1}, and $P_1 > 10\%$ at 1 mM $MgCl_2$ pH 8.0, 37°C.

N4 linker selection at 1 mM Mg^{2+} The result of the initial phase of selection at 20 mM Mg^{2+} is given in Figure 4a (showing generation 4). This population was directed through further rounds of selection at reduced Mg^{2+} concentration producing populations g6b, g6c, g6d (Figure 4b). Cleavage rate constants for a set of these molecules suggested that variants which show high activity at low Mg^{2+} concentration, comprise a discrete fraction of the entire set. The nature of this set was investigated by renewed selection, devised specifically to optimise identity between positions 10.1 and 11.1 (ie. L.1 - L.4).

Table 1 reveals that the composition of the linker region in minimised hammerheads is enormously significant. Most sequences will only support very low rates of cleavage. In the most highly evolved populations (g6c and g6d) the dramatic enrichment of molecules bearing the sequence motif G(10.1)NNNNC(11.1) was correlated to significantly improved cleavage rate constants for those populations (data not shown). However, kinetic analysis of individual members of these populations revealed that the G(10.1)NNNNC(11.1) motif, of itself, is insufficient to confer a robust cleavage phenotype (k_1 determined at 1 mM range from 0.04 - 1.4 min^{-1}). Rather it predisposed molecules towards having moderate to high cleavage kinetics (10 out of 17 molecules bearing this motif, displayed first phase k_1 values $\geq$ 0.5 min^{-1}, with only 4 having values $\leq$ 0.1, at 1 mM Mg^{2+}, pH 8.0, 37°C). The data indicated that only a very small number of linker permutations satisfy the requirement for very high cleavage activity. Assuming that a G at position 10.1 and a C at position 11.1 represents a kinetically favourable scaffold, we prepared a new randomised population of molecules, N4g0 (Figure 2c), comprising all 256 GNNNNC possibilities, and subjected this population to three rounds of selection with Mg^{2+} supply limited to 1 mM (Figure 5b). Amplified material from the resulting N4g1, N4g2, and N4g3, was cloned and sequenced. All the sampled molecules fit the description 5' NNHH in the linker region (L.1 - L.4) as originally suggested by the N18g6 consensus. The set includes some molecules which were previously observed plus additional representatives. Sequence data obtained from N4g3 allowed us to recognise three distinct classes of molecule within the NNHH

family (Figure 6). The high activity group identified among the N18g6 molecules, shown above the dotted line in Table 1, can be ascribed to these three classes. The data contained within the low Mg^{2+} evolved N4 populations therefore supports the finding from the low Mg^{2+} evolved N18g6 populations, that there is a discrete composition of linker which is required for high activity at low Mg^{2+} concentration.

Novel classes of RNA tetraloop Rate enhancement of hydrolysis of the scissile RNA phosphodiester bond in a miniribozyme/substrate complex, is presumably dependent on specific tertiary architecture. Effective linkers may exhibit structure which supports the catalytic core and whose sequence identity reduces the scope for misfolding. We would expect some linker structures to favour the adoption of an active conformation by reducing the number of steric possibilities, limiting the occurrence of events which constitute freedom to explore unreactive configurations. Others might suppress it by imposing torsion on the architecture or promoting an inactive structure, eg. through misfolding induced by inadvertent intramolecular homology.

Linkers supporting improved kinetic activity presumably act by providing a scaffold which will promote the correct folding of the conserved G-A base pair stack which constitutes domain II in the hammerhead crystal structures (29). The formation of this domain was proposed as the first of two tertiary folding movements required for progression to the catalytic ground state (21). Our data suggests that a G(10.1).C(11.1) base pair provides a key component of this scaffold. We propose that the other key component of an optimal scaffold in a miniribozyme is a linker sequence able to form a stable tetraloop between positions 10.1 and 11.1, ie. a structure which is able to turn around a narrow enough radius without creating torsion on the complex base-paired structure which stacks above it. This hypothesis is supported by the statistically high occurrence of the known tetraloop GNRA in the g6 populations (0.13 as compared to 0.03 expected random occurrence). The GNRA motif forms a sheared G-A base pair which enables it to reverse the direction of the RNA backbone within an extremely narrow radius (37). It is also thought to assist in the initiation of hairpin

folding (38-41). This folding dynamic in a miniribozyme would assist in providing a favourable equilibrium position, allowing a higher proportion of the molecules to be in an active conformation at any one time. However, there is evidence that some known stable tetraloops do not satisfy the requirement for high cleavage rates in a miniribozyme. For instance, the stable tetraloop CUUG, when incorporated into a G(10.1).C(11.1) miniribozyme, exhibited poor cleavage kinetics (28). Tetraloops are therefore effective within a context and do not necessarily transfer this effect when incorporated in a different context. The most effective molecules characterised here were GUUUUC (6.21), GGUAAC (6c10), and GUCUAC (6.14). Related molecules occurred at high frequency in the low Mg^{2+} selected N4 populations. Presumably these sequences are able to execute an effective turn through a narrow space without disrupting the correct base paired structure essential for optimum catalysis. Three classes of molecule were delineated in the N4g3 sequence data (Figure 6). All conform to the 5' NNHH consensus identified previously. Class I consensus (5' YRHH) includes UGAA (a known tetraloop (42). Class III has significant homology with the GNRA family. Class II does not resemble any known tetraloops, and therefore represents a novel and highly represented classification.

Tolerance to low Mg^{2+} concentration The hammerhead ribozyme appears to be dependent on Mg^{2+} for a structural transition within the ribozyme/substrate complex, which leads to the formation of the ground state. In the reaction, Mg^{2+} is thought to directly catalyse both the activation of the 2' nucleophile for attack on the phosphate centre, and the rate of departure of the 5' oxo leaving group. The composition of the linker in a miniribozyme could lower the Mg^{2+} dependence for any of these functions. Bassi and co-workers (19-21) have studied a structural equilibrium of the hammerhead as a function of Mg^{2+} concentration. High magnesium ion concentration favours the more active conformation (20). The biphasic kinetics we observe here suggest that there is a kinetic barrier between active and inactive forms of the ribozyme-substrate complex, and that the position of the equilibrium, as well as the

activity of the active form, is dependent on the nature of the linker between positions A9 and G12.

Crystal structures indicate a Mg^{2+} binding site at the apex of helix II in the hammerhead (22,29,30,43). Coordinating ligands indicated by Scott et al (30) are G8 (2'O), A9 (P1-O), G10.1 (N7), and G12 (N2). Whilst the miniribozyme is a novel structure, these positions are conserved (including G10.1 in the highly active species), therefore maintaining this binding potential. This binding site is suggested in all crystal structures for the conventional hammerhead, and has been proposed as a significant determinant of activity (29). The Kd of Mg^{2+} for this site will presumably be affected by the sequence of the linker region which lies immediately beneath it, and whose composition will affect the structure of the binding pocket. It is possible that some of the linkers provide optimal support for this function or that they provide an additional, or alternative high affinity Mg^{2+} binding site.

An alternative view is that stable and structurally appropriate tetraloop formation between positions 10.1-11.1 is able to facilitate a high K_d for Mg^{2+} in the catalytic core itself. This could involve the catalytic ion(s) or other specifically bound ions involved in structure. One proposed metal binding site involves A14 (20,44), and has a plausible role in influencing the formation of the core purine stack A9-G12 and G8-A13 (domain II, see Figure 1). Also, several studies (19,22,30,45-47) have indicated a metal binding site associated with G5 and A6 in the CUGA unpaired motif between helices I and II (positions 3-6) in the hammerhead, associated with the folding of domain 2. In structures by Scott et al (22,30,43) there are additional sites, one involving C3 and U4 and C17. This ion appears to coordinate with the 5'-O leaving group, and has a plausible role in catalysing its departure. A further ion is positioned to allow direct coordination with the pro-R oxygen adjacent to the scissile bond, and is plausibly implicated in activating the 2'-O nucleophile. In miniribozymes, the composition and proximity of the 10.1-11.1 linker must have considerable bearing on the structure of

this core region, and could therefore determine the Mg^{2+} sensitivity of the catalytic outcome.

KrMc10 and IL2Mc10 (miniribozymes with evolved linkers) versus KrRz and IL2Rz (hammerhead ribozymes with full length helix II) The data we have obtained suggests that the capacity for high cleavage rate constants in a miniribozyme is imparted by specific linker sequences. We next wished to see if a linker we identified could impart favourable kinetic properties when it was incorporated into a miniribozyme targeted to a different substrate. Miniribozymes with a 6c10 GGUAAC identity in the linker were chemically synthesised with 9 nucleotides in each hybridising arm to target a 19mer human IL2 mRNA substrate (IL2S19) and a 17mer *Drosophila melanogaster* Krüppel mRNA substrate (KrS17). These substrates provided targets with 9 nucleotides (IL2S19) and 8 nucleotides (KrS17) arranged symmetrically on either side of the unpaired C adjacent to the scissile phosphodiester bond. Rate constants for these miniribozymes were measured at 1 mM and 10 mM $MgCl_2$ (pH 7.6, 37°C) and compared to those obtained for hammerhead ribozymes (KrRz and IL2Rz). KrRz was constructed with 10 nucleotides in each arm and IL2Rz was constructed with 9 nucleotides in each arm. Both KrRz and IL2Rz had 4 Watson-Crick base pairs in helix II.

Table 2 illustrates that the favourable kinetic traits exhibited by the 6c10 motif were equally observed in both the Krüppel and IL2 substrate backgrounds. KrMc10 and IL2Mc10 (miniribozymes with 6c10 linkers) were also more active than similarly constructed molecules with full length helix II. When cleaving gene length substrates, optimum results for both minimised and conventional hammerhead ribozymes are obtained when helix I and helix III are each ~9 bp (48,49). However, cleavage rate constants for conventional hammerheads on short substrates vary markedly in response to the length of helix I, and appear to be highest when helix I is 5-6 bp (31). Rate constants for miniribozymes appear to be less susceptible to changes in arm length (32). When helix I is 9 bp or 8 bp, rate constants for the hammerhead ribozyme

were poor as compared to the optimised miniribozymes described here. Furthermore, the difference between miniribozymes and hammerheads becomes greater when Mg^{2+} concentration is low.

Relation to other work The most recent communication involving hexanucleotide replacement of helix II, reported G(10.1)CGNGC(11.1) as a highly active set of motifs identified using *in vitro* selection (50). Rate constants for the most active motif (GCGUGC) were 0.64 min^{-1} (0°C, 1 mM $MgCl_2$, pH 8.0), and 0.23 min^{-1} (37°C, 1 mM $MgCl_2$, pH 8.0). Zillmann et al clearly indicate that C(L.1).G(L.4) is a preferred identity according to their scheme of selection. In the context of the bacterial rRNA CUUG tetraloop, the C-G form a buckled base pair allowing an efficient and stable turn in the RNA backbone (51). However, this motif has been indicated to provide an inadequate scaffold for a miniribozyme (28), and our results suggests that a G at position L.4 is unfavourable. The differences between the Zillmann result and those reported here is no doubt due to the design of the selection protocol. Their study reports that selection was conducted at 0°C and with very long helices I and III. The observation that the GCGUGC molecule cleaves its substrate more rapidly at a lower temperature is unusual. This perhaps illustrates the need to formulate conditions of selection which are consistent with the intended use of the molecule. At a physiologically relevant temperature (37°C) the molecules we have identified in this study cleave between 2 and 10 times more rapidly than those identified by Zillmann et al. Previous studies have demonstrated that the replacement of stem-loop II in the hammerhead with a six nucleotide linker resulted in a reduction of catalytic activity. Tuschl and Eckstein (28) report an order of magnitude reduction in k_{cat} (ie. 3.1 min^{-1} to 0.3 min^{-1}) when a 4 bp helix II was replaced by the linker sequence G(10.1)CUUGC(11.1). Similarly, Long and Uhlenbeck (52) report a 10-fold decrease in k_{cis} (1.0 to 0.09 min^{-1}) and k_{cat} (1.5 to 0.12 min^{-1}) when they compare a 4 bp helix II molecule to G(10.1)UUUGC(11.1).

Table 1. Kinetic paramaters for selected ribozymes at 1 mM and 10 mM $MgCl_2$. Substrate was IL2biosS (29mer RNA).

	Linker Position			Cleavage Kinetics 1m*M* (10 m*M*) [a] $MgCl_2$, pH 8.0, 37°C		
Ribozyme	10.1	L.1-L.4	11.1	k_1 [b]	P_1 [c]	P∞ [d]
6.18		CACC	[e]	0.9	13.0	81.3
6.21		UUUU		0.9(4.8) [a]	47.3(59.5)	77.3(91.3)
6.24		UUAA		1.0(1.9)	36.6(51.8)	84.4(80.8)
6.17		UCCA		1.1(5.5)	14.5(23.3)	83.9(62.1)
6.14		UCUA		0.5(2.3)	36.1(50.3	70.8(77.6)
6c10		GUAA		0.6(3.0)	44.9(41.5)	81.4(76.6)
6.22		CAUA		0.9	8.8	72.8
6.19		CUAA		0.5	5.2	65.3
6.11		CAAA		1.1(4.3)	4.4(37.3)	98.0(88.9)
6.7		CUAA		1.4	2.3	54.4
6.2		ACCA		0.7	7.5	74.0
6.5		GGGA		0.07	-	79.7
6.10		GGGA		0.04(0.4)	-	75.5
6.16		GCAA		0.32(2.7)	7.2(38.4)	88.1(82.6
6.20		CUCC		0.9	5.1	37.0
6.23		AAAC		0.02	-	76.6
4.14		AAU		0.04(0.6)	- (11.6)	68.0(73.6)
4.13	G	UAU	U	0.02(0.7)	- (7.9)	73.7(69.4)
6b9	A	UAU	U	0.01	-	67.2
6.3	A	GAAA	U	1.1(1.7)	6.5(31.4)	43.2(55.8)
6.4	A	UUUU	G	0.007	-	32.1
6.12	C	UUGG	A	0.01(0.04)	-	49.1(64.2)
4.31	U	UAU	U	0.007	-	44.7
6.6	U	UUGG	U	0.01	-	97.1
4.21	U	CCAC	U	0.006	-	67.7
6.9	U	AUUU	U	0.5(1.0)	6.6(52.8)	76.4(93.8)
6.13	U	AUUA	U	0.009(1.1)	- (4.2)	76.4(94.2
6d10	U	GGUA	U	0.008	-	77.4

[a] values in parenthesis () were obtained using 10 mM $MgCl_2$ in the cleavage reaction.

[b] k_1 = first order rate constant (min^{-1}).

[c] P_1 = extent of the first phase (percentage cleaved).

[d] $P\infty = P_1 + P_2$, estimated endpoint (percentage cleaved).

[e] highlighted block are those molecules that a G at position 10.1 and a C at position 11.1.

Table 2 Kinetic parameters for cleavage of IL2S19 by IL2Mc10 (GGUAAC linker) and IL2Rz, and KrS17 by KrMc10 (GGUAAC linker) and KrRz, at 1 mM and 10 mM $MgCl_2$.

Ribozyme	10.1 - 11.1	Cleavage Kinetics 1m*M* (10 m*M*) [a] $MgCl_2$, pH 7.6, 37°C		
		k_1 [b]	P_1 [c]	$P\infty$ [d]
IL2Mc10	GGUAAC	1.3(3.6) [a]	22.2(60.0)	74.8(75.6)
IL2Rz	GUCCUUUUGGAC	0.3(3.9)	- (27.9)	31.7(44.2)
KrMc10	GGUAAC	1.3(3.8)	21.0(54.9)	75.4(75.6)
KrRz	GUCCUUUUGGAC	0.1(2.4)	- (39.5)	55.4(59.3)

[a] values in parenthesis () were obtained using 10 mM $MgCl_2$ in the cleavage reaction.

[b] k_1 = first order rate constant (min^{-1}).

[c] P_1 = extent of the first phase (percentage cleaved).

[d] $P\infty = P_1 + P_2$, estimated endpoint (percentage cleaved).

Conclusion

The results here show that there is no implicit or necessary reduction of activity associated with the minimisation of helix II to a single Watson-Crick base pair. The activity of a miniribozyme is dependent on linker composition. Rate constants in excess of those observed for conventional hammerhead ribozymes can be achieved by appropriately engineered linker motifs. The composition of this linker region has been thoroughly assayed in this study by means of *in vitro* evolution. Three classes of linker design have been elucidated via *in vitro* selection using low Mg^{2+} concentrations. The high activities displayed by some of these miniribozymes, and the transferability of these observations into different substrate backgrounds, has resulted in generalised designs for oligonucleotide reagents that retain high catalytic activity at physiological concentrations of Mg^{2+} ion. Whilst the relative activities of miniribozymes and more conventional hammerheads will almost certainly vary with the length of helix I, the superiority of the miniribozyme at a physiological concentration of Mg^{2+}, using helix lengths devised for optimum results on gene length substrates, suggests that miniribozymes offer a first choice design for *in vivo* studies.

Persons skilled in this art will appreciate that variations and modifications may be made to the invention as broadly described herein, other than those specifically described without departing from the spirit and scope of the invention. It is to be understood that this invention extends to include all such variations and modifications.

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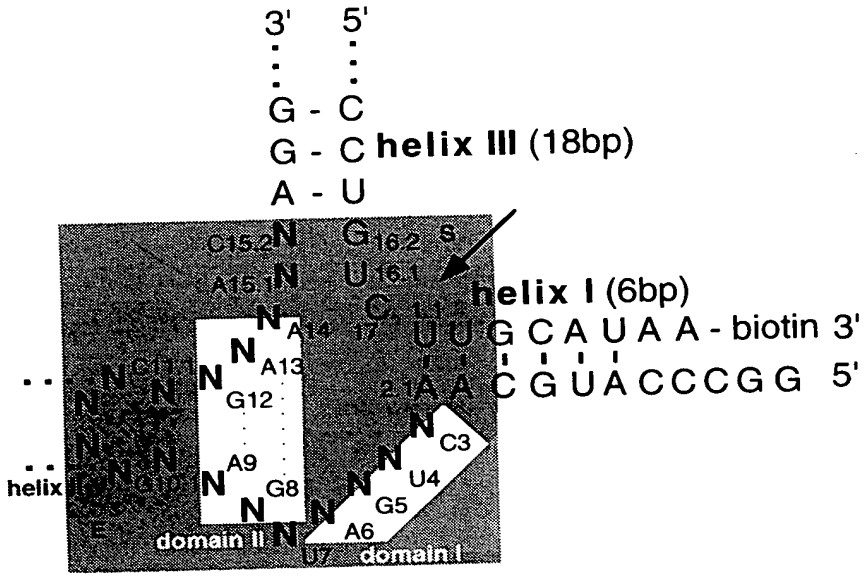
Dated this 24th day of December, 1998.

Commonwealth Scientific and Industrial Research Organisation

By its Patent Attorneys

Davies Collison Cave

figure 1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5'
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G - C
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A - U
helix I (6bp)
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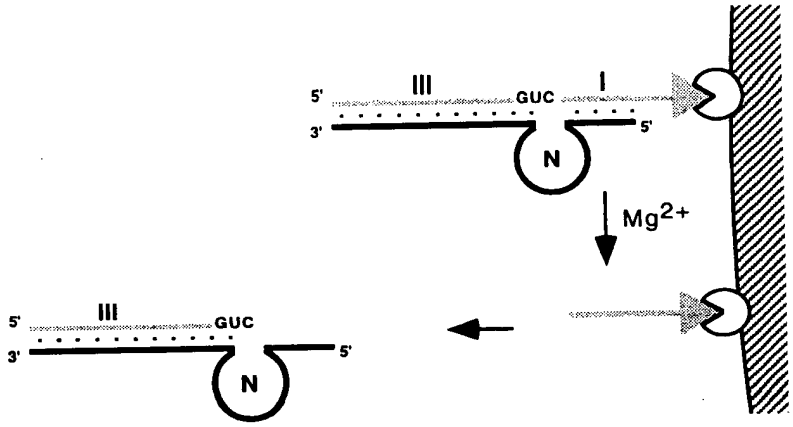
A A C G U A C C C G G 5'
domain II
domain I
helix II

a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I
3'
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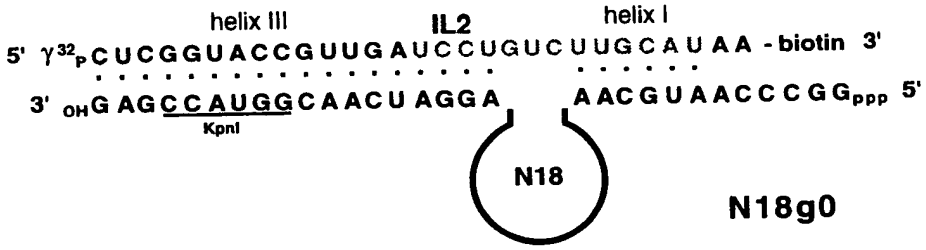
Mg2+
5'
III
GUC
3'
5'
N

b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helix I
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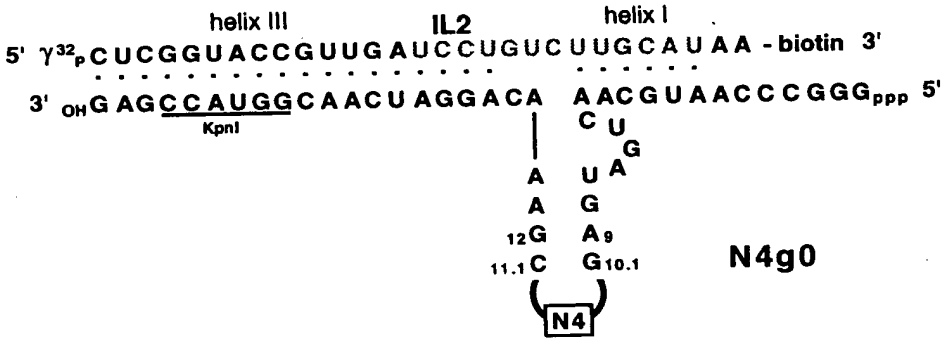
KpnI
N18
N18g0

c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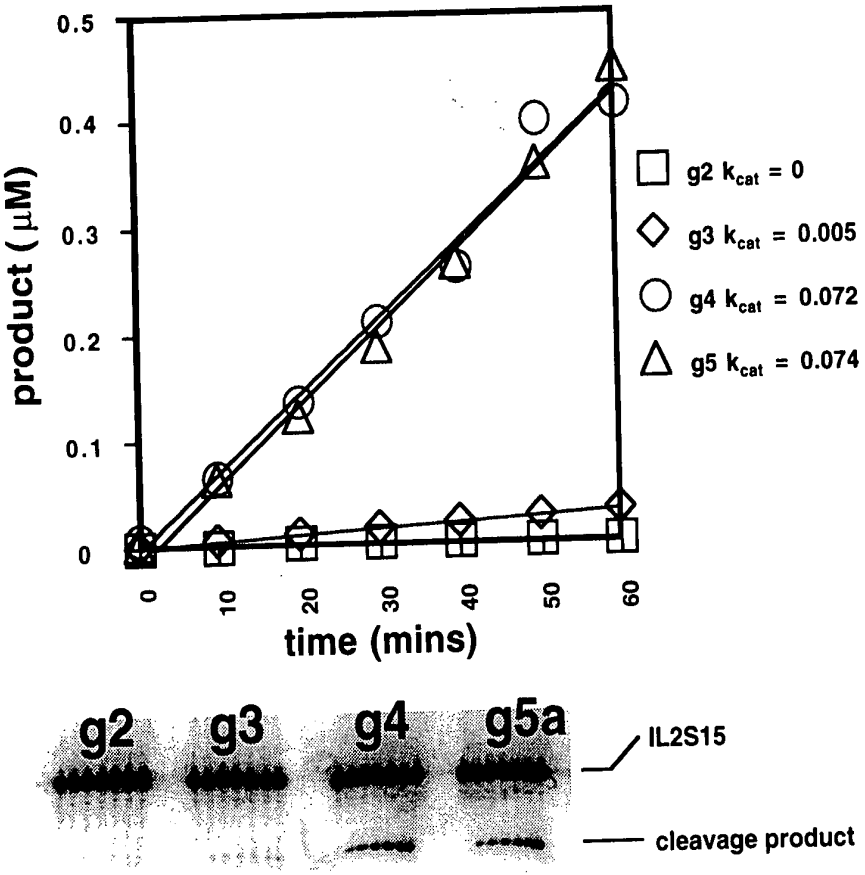

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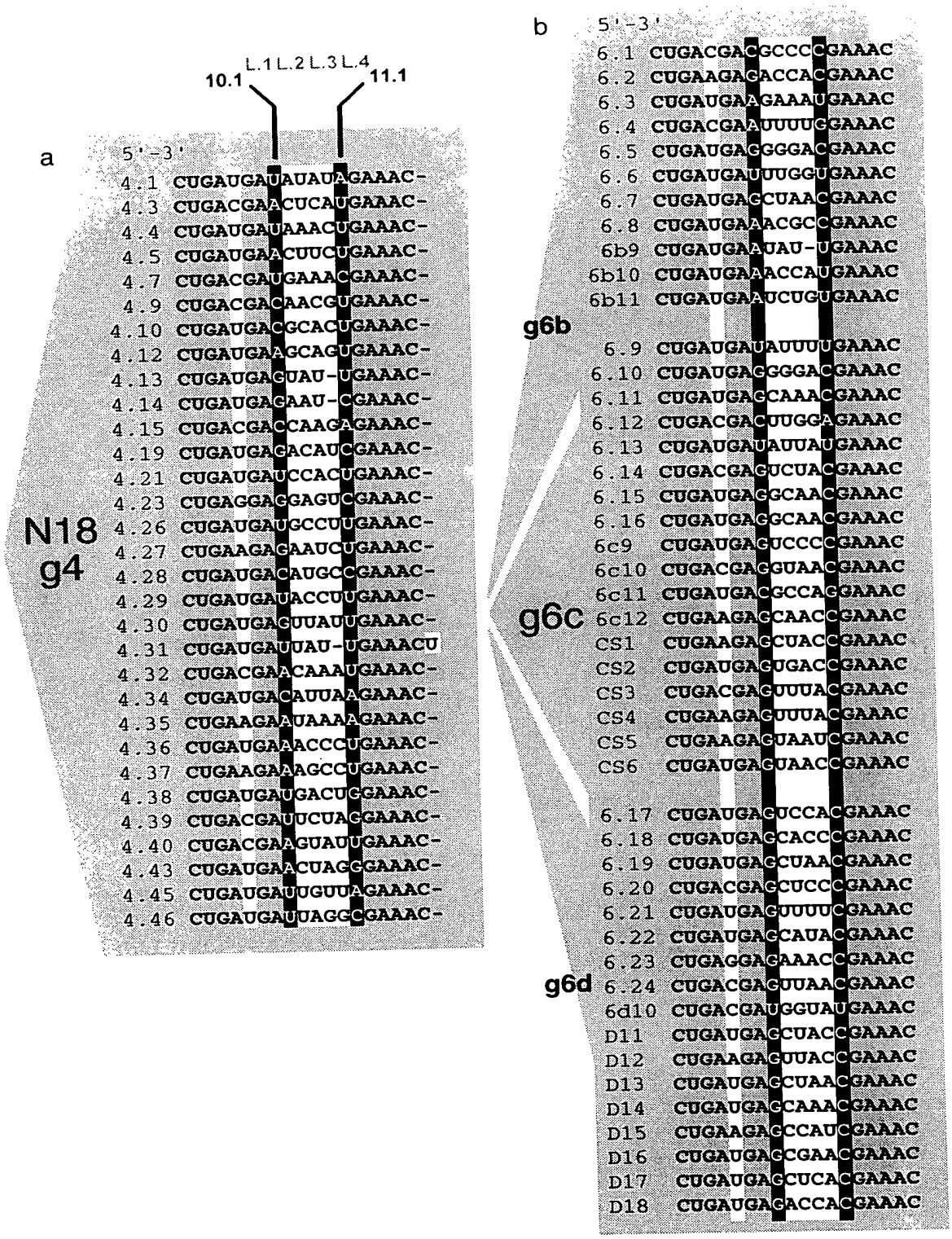

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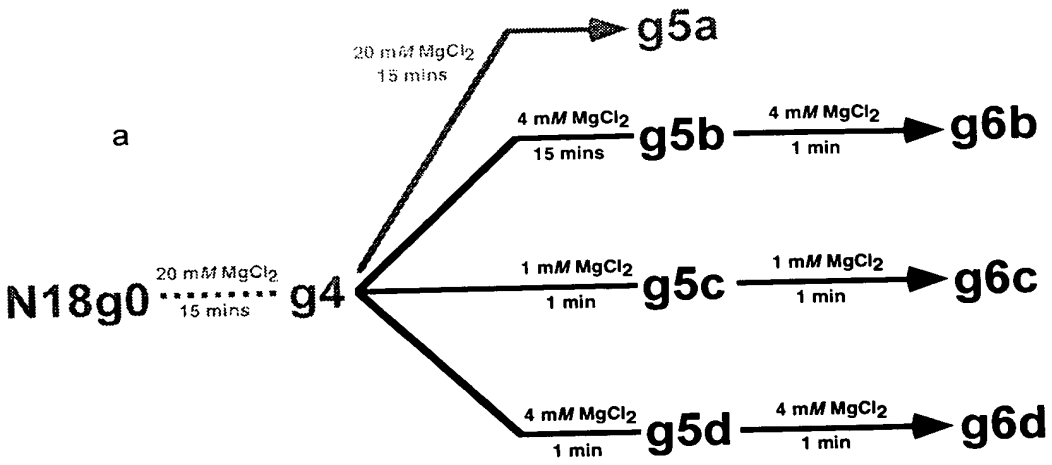
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g6b
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1 min
g5c
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1 min
g5d
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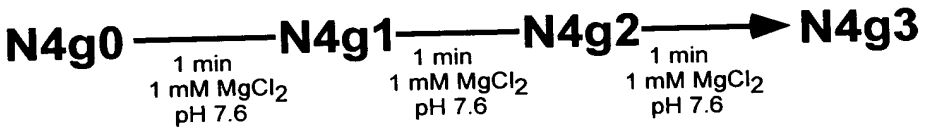
1 min
g6d

b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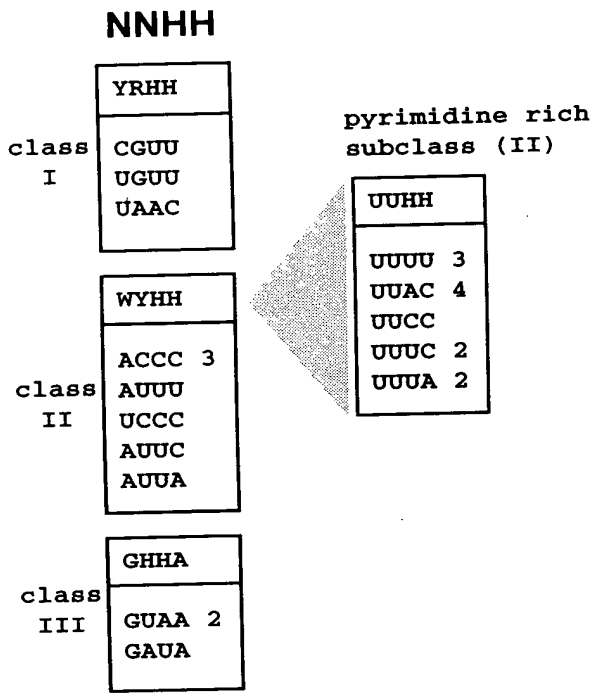
pH 7.6
N4g3

figure 5

figure 6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AUUC
AUUA
GHHA
class III
GUAA 2
GAUA
pyrimidine rich subclass (II)
UUHH
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UUAC 4
UUCC
UUUC 2
UUUA 2